Assay for fluorescein diacetate hydrolytic activity: Optimization for soil samples

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Abstract

With the increased interest in integrated soil bioecosystem studies, there is a need to have a method of measuring overall microbial activity potential. Hydrolysis of fluorescein diacetate [$3',6'$-diacetylfluorescein (FDA)] has been suggested as a possible method because the ubiquitous lipase, protease, and esterase enzymes are involved in the hydrolysis of FDA. Following hydrolysis of FDA, fluorescein is released and can be measured spectrophotometrically. Our objective was to optimize the assay for FDA hydrolytic activity in soil samples and determine the kinetic parameters involved in this reaction. The optimized method involves extraction and quantitative measurement of the fluorescein released when 1.0 g of soil is incubated with 50 ml of 60 mM Na–phosphate solution (buffered at pH 7.6) at 37 °C for 3 h. Results showed that FDA hydrolysis was optimum at buffer pH 7.6 and the soil enzymes were denatured at temperatures above 60 °C. Three soils were used to optimize this method: Heiden clay, Raub silt loam, and Cecil sandy loam. This procedure is simple, precise, and can be used in commercial soil testing laboratories to determine general microbial activity and as a soil quality indicator.
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1. Introduction

During the past few years, interest in the size and activity of the soil microbial biomass is increasing, partly due to the importance of this information in integrated bio-ecosystem and global change studies. Total microbial activity provides a general measure of organic matter turnover in natural habitats as about 90% of the energy in the soil environment flows through microbial decomposers (Heal and McClean, 1975).

Fluorescein diacetate [$3',6'$-diacetylfluorescein (FDA)] can be used to measure microbial activity in soils (Brunius, 1980; Lundgren, 1981; Schnürer and Rosswall, 1982). Fluorescein diacetate is hydrolyzed by a number of different enzymes, such as proteases, lipases, and esterases. The equation of the reaction is:
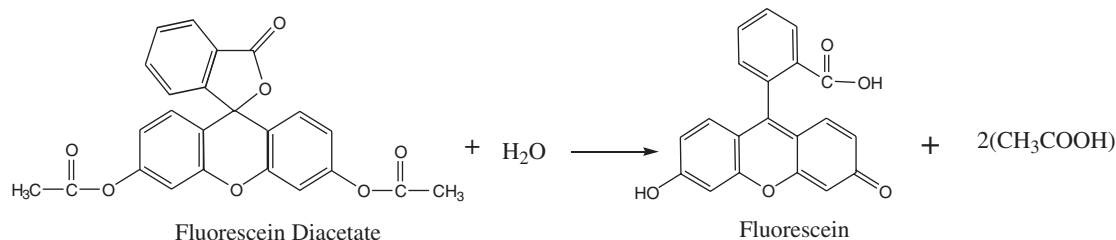
Fluorescein Diacetate $+$ H$_2$O $\longrightarrow$ Fluorescein $+$ 2(CH$_3$COOH)

The product of this enzymatic reaction is fluorescein, which can be visualized within cells by fluorescence microscopy (Lundgren, 1981). Fluorescein released in soil can also be measured by spectrophotometry (Swisher and Carroll, 1980; Schnürer and Rosswall, 1982). A search of

the scientific literature revealed little information on the factors affecting the FDA hydrolysis in soils (Schnürer and Rosswall, 1982; Adam and Duncan, 2001). The Schnürer and Rosswall method, which has been to date the most frequently used method to determine FDA hydrolysis activity in the soil, was developed for pure microbial cultures, and was not originally optimized for soil samples. Pure culture work does not take into account the influence of the soil itself on the methodology, which may adsorb substrate or introduce interfering factors such as Fe or Al. Additionally, the fluorescein diacetate immobilized within a soil environment would come from many microbial and plant species. Several investigators (private communications) have found it difficult to achieve reproducible results with the existing method.

The objective of this investigation was to develop a simple, rapid, and precise method to assay fluorescein diacetate hydrolysis, specifically optimized for soil, which could be used as a biochemical/biological indicator of soil quality and to determine the kinetic parameters involved in this reaction. The optimized procedure we present here is different in many respects from the procedure of Adam and Duncan (2001).

2. Materials and methods

2.1. Soils

Three surface soil samples, selected to obtain a wide range in pH, organic C, total N and texture, were used in the development of the method (Table 1). The Heiden clay was sampled from the USDA-ARS Grassland, Soil, and Water Research Laboratory field station at Reisel, Texas; and is dominated by smectite clays with some quartz and calcite (Reichert and Norton, 1994). Raub silt loam was sampled from the Purdue University Agronomy Center for Research and Education (ACRE) in West Lafayette, Indiana. Cecil sandy loam was sampled from the USDA-ARS Southern Piedmont Conservation Research Center in Watkinsville, Georgia; it is dominated by kaolinite clays with some quartz and hydroxy-interlayer vermiculite (Reichert and Norton, 1994). The samples were air-dried and sieved (2.0 mm).

An additional five soils were used for testing the precision of the method. The Barnes, Mexico, Miami, Portneuf, and Tifton soils (Table 1) had been previously collected, air-dried, sieved (2.0 mm), and stored at the USDA-ARS National Soil Erosion Research Laboratory until use.

2.2. Reagents

Sodium phosphate buffer was prepared by dissolving 22.74 g sodium phosphate tribasic ($Na_3PO_4 \cdot 12H_2O$) in approximately 700 ml of deionized water in a 1 l volumetric flask. To test the effects of pH on FDA hydrolytic activity,

the pH was adjusted to 7.2, 7.4, 7.6, 7.8, or 8.0 with 1 M HCl. Deionized water was added to adjust the final volume to 1 l.

The FDA substrate stock solution ($C_{24}H_{16}O_7$, Sigma-Aldrich Chemical Co., Milwaukee, WI) was made by dissolving 5 mg FDA in 10 ml of reagent-grade acetone for a final solution concentration of 12.01 μM FDA ml^{-1}.

A fluorescein standard stock solution was prepared by dissolving 10 mg fluorescein ($C_{20}H_{12}O_5$, Sigma-Aldrich Chemical Co., Milwaukee, WI) into 10 ml of reagent-grade acetone in a 50 ml volumetric flask. The final volume was adjusted to 50 ml with sodium phosphate buffer (pH 7.6) for a 602 μM solution.

2.3. Initial method

Various properties of the FDA hydrolytic activity in soils were studied. These factors included time of incubation, optimum pH buffer, temperature of incubation, substrate concentration, and influence of shaking. All optimization procedures were based on a preliminary method we had developed earlier. Testing of the various components to determine optimal levels were carried out individually, leaving all other components as they were in the preliminary method.

For the initial method, a 1 g soil sample (air-dried, sieved <2.0 mm) was placed into a 125 ml Erlenmeyer flask. Fifty milliliter of 60 mM sodium phosphate buffer (pH 7.6) was added followed by 0.50 ml of FDA substrate solution for a final substrate concentration of 6.0 μM. The flasks were then placed on an orbital shaker for 3 h at 24 °C. Then 2 ml of acetone were added and the suspension was swirled to terminate FDA hydrolysis. Thirty milliliter of the soil suspension was transferred to 50 ml centrifuge tubes and the soil suspension was centrifuged at 8000 rev min^{-1} (8820g) for 5 min in a refrigerated centrifuge (Beckman J2-HS). The supernatant was then filtered through a Whatman No. 2 filter paper. The filtrate was transferred to a colorimeter tube and the absorbance was measured on a spectrophotometer set at a wavelength of 490 nm.

2.4. Optimization procedures

To determine the optimum buffer pH, 60 mM sodium phosphate buffer solutions were prepared at pH of 7.2, 7.4, 7.6, 7.8, or 8.0. Buffer pH was modified by adding 1.0 M HCl to the sodium phosphate buffer solution. The choice of 60 mM of sodium phosphate, buffered at pH 7.6 and 24 °C incubation was based on the method of Schnürer and Rosswall (1982).

Optimization of incubation time was determined by incubating samples for 1, 2, 3, 6, or 12 h. Temperature variations were at 24, 37, 50, 60, and 70 °C.

To determine the impact of shaking during incubation on FDA activity, samples were shaken (200 rev min^{-1}) in an

Table 1
Properties of the tested soils. Soil classification is based on the US Soil Taxonomy system (Natural Resources conservation Service, 2005)

Soil series	Soil classification	Textural class	Location (State)	pH	Organic C (g 100 g^{-1} soil)	Total N (g 100 g^{-1} soil)	Clay (g 100 g^{-1} soil)	Sand (g 100 g^{-1} soil)
Barnes	Fine-loamy, mixed, superactive, frigid Calcic Hapludolls	Loam	MN	6.2	2.48	0.23	17	49
Cecil[a]	Fine, kaolinitic, thermic Typic Kanhapludults	Sandy loam	GA	5.3	0.47	0.06	13	71
Heiden[a]	Fine, smectitic, thermic Udic Haplusterts	Clay	TX	7.8	2.10	0.17	57	13
Mexico	Fine, smectitic, mesic Aeric Vertic Epiaqualfs	Silt Loam	MO	6.9	2.43	0.25	26	5
Miami	Fine-loamy, mixed, active, mesic Oxyaquic Hapludalfs	Silt Loam	IN	5.9	1.90	0.19	19	10
Portneuf	Coarse-silty, mixed, superactive, mesic Duri-nodic Xeric Haplocalcids	Silt Loam	WA	8.1	0.84	0.07	20	18
Raub[a]	Fine-silty, mixed, super-active, mesic Aquic Argiudolls	Silt Loam	IN	5.0	1.34	0.15	21	16
Tifton	Fine-loamy, kaolinitic, thermic Plinthic Kandiudults	Loamy Sand	GA	5.7	0.51	0.015	11	80

[a] Soils used for development of optimized method.

environmental shaker or left in a static (unshaken) condition in the environmental shaker.

Optimization of substrate concentration was determined by varying the stock substrate concentrations to 1.2, 2.4, 3.6, 4.8, and 7.2 mM fluorescein diacetate prepared in 10 mL of reagent-grade acetone (5, 10, 15, 20 or 30 mg FDA added to 10 ml acetone, respectively). This corresponded to substrate concentrations of 11.9, 23.8, 35.7, 47.6, and 71.3 μM in the incubating solution. Once the final method was developed, this test was run again using the final optimized method.

For all stages of method development, samples were run in triplicate, and the mean, standard deviation, and coefficient of variation were determined.

2.5. Final method

This method for assay of FDA hydrolytic activity in the soil was developed after all the factors involved in the assay had been optimized: place 1.0 g of air-dried soil in a 125 ml Erlenmeyer flask. Add 50 ml of 60 mM sodium phosphate buffer, pH 7.6 and 0.50 ml of 4.9 mM FDA lipase substrate solution (20 mg FDA lipase substrate in 10 ml acetone). Stopper the flask and swirl for a few seconds to mix the contents. Place in an incubator for 3 h at 37 °C. Then add 2 ml of acetone to the suspension and swirl to mix the contents and terminate FDA hydrolysis. Transfer about 30 ml of soil suspension to a 50 ml centrifuge tube and centrifuge (Beckman J2-HS) at 8000 rev min^{-1} (8820g) for 5 min. Filter the supernatant through a Whatman No. 2 filter paper. Transfer the filtrate to a colorimeter tube and measure the absorbance on a spectrophotometer (Beckman DU-64) set at a wavelength of 490 nm.

Controls should be performed with each soil analyzed to measure the color not derived from the hydrolysis of FDA. To perform controls, follow the procedure described for the enzyme assay, but add 0.50 ml acetone instead of the fluorescein diacetate lipase substrate solution. Run blanks (samples with no soil, just the reagents), as slight color development occurs spontaneously at 37 °C and should be subtracted from the results obtained.

Calculate the concentration of fluorescein released by reference to a standard curve from the results obtained with standards containing 0.03, 0.1, 0.3, and 0.5 mg of fluorescein. To prepare the working standards, pipette 0.15, 0.5, 1.5, and 2.5 ml of the fluorescein standard stock solution into 50 ml volumetric flasks. Bring to volume with sodium phosphate buffer (pH 7.6) and then add 2.5 ml of acetone, matching the matrix of the samples. Measure the absorbance at a wavelength of 490 nm on a spectrophotometer. The standard curve is linear in this range and covers the normal range of FDA activity in soils. If the absorbance of the sample exceeds the limits of the standard curve, dilute the filtrate with sodium phosphate buffer until the absorbance is within the limits of the standard curve.

2.6. Precision of method

The precision of the final method was determined using the optimized procedure on eight soils of varying characteristics sampled from the USA (Table 1). Two replicate samples (with a control) were run on three separate days, for a total of six replicates. The mean, standard deviation, and coefficient of variation were determined from these replicate samples.

2.7. Enzyme kinetics

The temperature coefficients (Q_{10}), activation energy (E_a), Michaelis constant (K_m), and maximum rate (V_{max}) were determined using the optimized method. Measurements for determining the Q_{10} were taken at 20, 30, and 40 °C. The K_m and V_{max} values were determined using the Lineweaver–Burke transformation of the Michaelis–Menten equation while the activation energy was determined using the Arrhenius equation.

3. Results

The method developed for the assay of fluorescein diacetate (FDA) hydrolysis in soils is based on the quantitative extraction and colorimetric determination of fluorescein released in soils. Systematic studies of factors affecting the release of fluorescein during incubation of soil with a buffered solution aided optimization of this assay. Factors included pH, incubation time, incubation temperature, shaken vs. static incubation, and substrate concentration. The initial method used for the testing of the factors was primarily based on the widely used Schnürer and Rosswall (1982) method. After the development of the final method, the precision of the method was tested and some of the kinetic parameters were determined.

3.1. Optimization of method

Using the initial method, the pH of the buffering solution was varied between 7.2 and 8.0. Optimal activity of FDA hydrolytic soil enzymes was observed at pH 7.6 for Cecil sandy loam and Raub silt loam (Fig. 1), with activity levels at other pH values not being significantly different from one another. Heiden clay showed optimum activity at a pH of 7.6 up to 8.0. The Heiden soil also showed the most variability within a pH treatment. The buffering capacity of solution was sufficient to keep the pH at 7.6 for the duration of the experiment.

For the three soils evaluated, the release of fluorescein during FDA hydrolysis increased through 12 h (Fig. 2). The Heiden soil showed a break from linearity at about 3 h whereas the Cecil and Raub soils showed a nearly linear response throughout the 12 h incubation range tested.
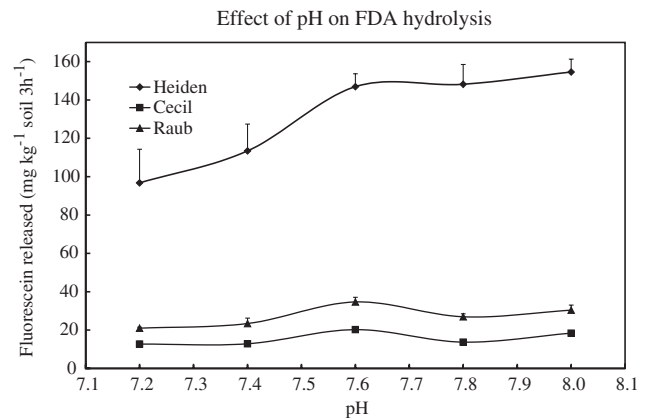

Fig. 1. Effects of the buffer pH on the release of fluorescein during FDA hydrolytic activity in Heiden, Cecil, and Raub soils. Points represent the mean of 3 replicates; error bars represent standard deviation.

A study of FDA hydrolytic activity in soils as a function of temperature showed increasing activity up to 60 °C under the conditions of the preliminary assay (Fig. 3). Heiden expressed the greatest activity followed by the Raub and Cecil soils, respectively. Activity fell sharply between 60 and 70 °C in all soils tested. At the higher activity levels, greater variability for a given treatment was seen.

Use of 2 ml acetone to halt the reaction did not result in complete stoppage of the hydrolysis reaction (data not shown), but did slow it down sufficiently to allow readings within 30 min with no significant differences in the spectrophotometer readings. After 30 min, noticeable and significant differences began to be seen.

The Cecil sandy loam and the Raub silt loam soils responded with higher activity when the samples were not shaken during incubation as opposed to being shaken at 200 rev min^{-1}. The Heiden clay soil exhibited no difference between static and shaken incubations (Fig. 4).

For a valid assay of enzymatic activity, it is necessary to ensure that the enzyme substrate concentration is not limiting the reaction rate during the assay procedure. A
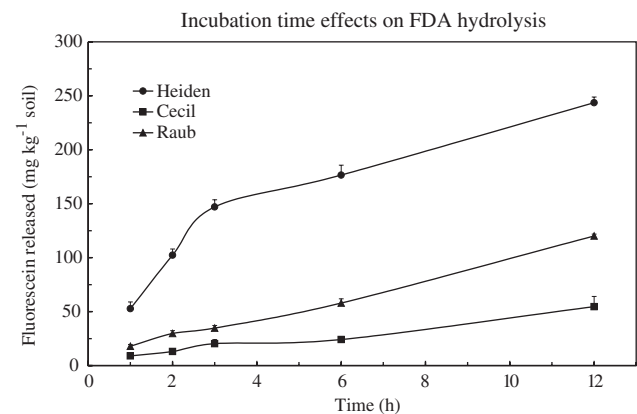

Fig. 2. Effects of incubation time on release of fluorescein during FDA hydrolysis in Heiden, Cecil, and Raub soils. Points represent the mean of three replicates; error bars represent standard deviation.
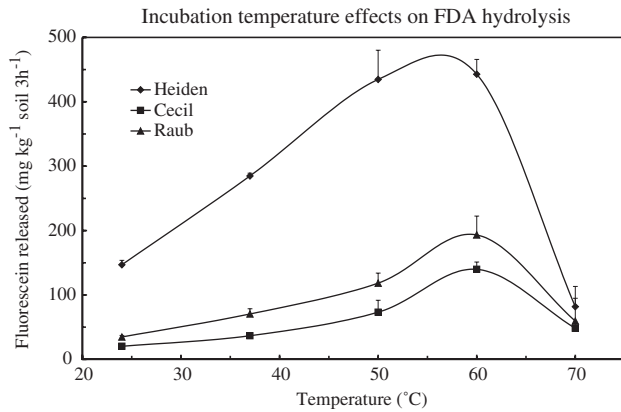
Fig. 3. Effects of incubation temperature on the release of fluorescein during FDA hydrolytic activity in the Heiden, Cecil, and Raub soils using the initial method. Points represent the mean of 3 replicates; error bars represent standard deviation.

Fig. 5. Effects of substrate concentration on release of fluorescein during FDA hydrolytic activity in Heiden, Cecil, and Raub soils using the initial procedure (24 °C, 3 h shaken incubation). Points represent the mean of three replicates; error bars represent standard deviation.

study of the effect of varying substrate concentration showed that substrate concentration was satisfactory for the FDA hydrolysis assay under the conditions of the initial method (shaken at 24 °C, Fig. 5). It also showed that soils with low activity did not respond much to changes in the substrate concentration range evaluated, yet Heiden clay with a high activity rate, showed a considerable response to substrate concentration variation. Fluorescein diacetate hydrolytic activity in Heiden clay increased with substrate concentration up to 23.8 μM and then decreased with increasing concentrations starting with 35.7 μM.

We re-evaluated the effects of substrate concentration using the optimized method (static incubation at 37 °C, pH 7.6; Fig. 6), as we were having difficulty obtaining repeatable values for the Heiden clay, which exhibits high FDA hydrolytic activity. Under the conditions of the new method, substrate concentration was varied, and the optimum substrate concentration appeared to be 47.6 μM

for the Raub and Cecil soils, while the Heiden clay was just beginning to plateau at this concentration. It was expedient not to use a final substrate concentration greater than 47.6 μM because at higher concentrations, a precipitate began to form.

3.2. Precision of method

The final method (static incubation at 37 °C with a 47.6 μM substrate concentration) was tested for precision (Table 2) using the three test soils as well as five other soils representing a broad range of soil characteristics (Table 1). Means for FDA hydrolytic activity, measured on three separate occasions for each soil, ranged from 66.1 mg fluorescein kg^{-1} soil $3h^{-1}$ for a Raub soil to 226.3 mg fluorescein kg^{-1} soil $3h^{-1}$ for a Heiden soil (Table 2). The percent standard deviation for the eight soils tested with this final method averaged 3.2% and ranged from 2.2 to 4.4%.
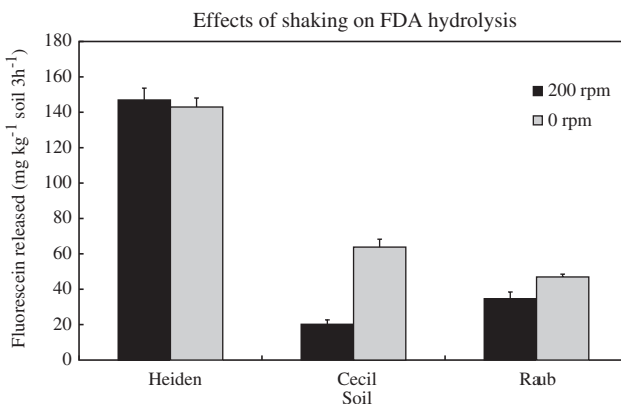

Fig. 4. Effects of mechanical shaking on release of fluorescein during FDA hydrolytic activity in the Heiden, Cecil, and Raub soils using the initial method. Mechanical shaking took place on an orbital shaker at 200 rev min^{-1}. Values represent the mean of three replicates; error bars represent standard deviation.
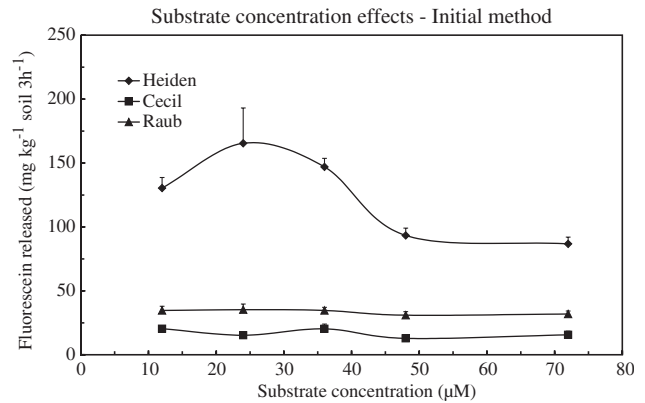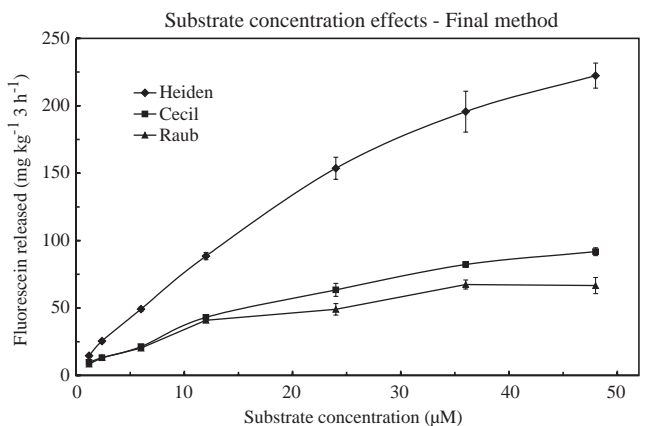

Fig. 6. Effects of substrate concentration on release of fluorescein during FDA hydrolytic activity in the Heiden, Cecil, and Raub soils using the final, optimized procedure (37 °C, 3 h static incubation). Points represent the mean of three replicates; error bars represent standard deviation.

Table 2
Precision of the final, optimized method of analysis for FDA hydrolytic activity

| Soil series | Milligram fluorescein released kg^{-1} soil 3 h^{-1} | | | |
	Range[a]	Mean	SD	CV (%)
Barnes	131.1–142.3	135.8	5.1	3.7
Cecil	88.4–95.5	92.3	3.0	3.2
Heiden	216.2–27.1	226.3	9.9	4.4
Mexico	157.1–171.2	161.4	5.1	3.2
Miami	154.2–165.0	158.9	4.3	2.7
Portneuf	93.5–100.0	96.7	2.9	3.0
Raub	64.4–68.0	66.1	1.4	2.2
Tifton	79.3–85.5	82.5	2.4	2.9

SD, standard deviation; CV, coefficient of variation.

[a] Range of six replicates, two replicates per day over 3 days.

Heiden displayed the largest variation as well as the highest FDA hydrolytic activity.

3.3. Kinetic parameters

The FDA hydrolysis reaction increased in activity in the three test soils by an average factor of 1.52 between 20 and 40 °C (Table 3).

The Arrhenius equation is used to represent the dependency of enzyme reactions on temperature as follows:

$$k = A \exp\left(\frac{E_a}{RT}\right) \qquad (1)$$

where k is the rate constant, A is the pre-exponential factor, E_a is the activation energy, R is the universal gas constant, and T is the temperature in degrees Kelvin. Expressing it in natural logarithmic form, the Arrhenius equation becomes:

$$\ln k = \ln A - \frac{E_a}{RT}. \qquad (2)$$

The Arrhenius equation plot for FDA hydrolytic activity in the test soils was linear between 20 and 40 °C (Fig. 7). The activation energy can be calculated from a plot of $\ln k$ versus $1/T$ where the activation energy is the negative slope of the line (Fig. 7; Chang, 2000). The activation energies for the soils used in this study ranged between 27.2 and 35.0 kJ mol^{-1} (Table 3).

By plotting the reaction velocity against the substrate concentration, the maximum velocity (V_{max}) and Michaelis

Table 3
Activation energies (E_a) and temperature coefficients (Q_{10}) of fluorescein diacetate hydrolytic activity in soils

| Soil series | E_a (kJ mol^{-1}) | Q_{10}[a] of the temperature indicated | | |
		30 °C	40 °C	Mean
Cecil	27.2	1.53	1.33	1.43
Heiden	35.0	1.67	1.50	1.58
Raub	32.7	1.63	1.45	1.54

[a] $Q_{10} = \frac{\text{FDA hydrolysis at specific temperature, } T}{\text{FDA hydrolysis at specific temperature, } T-10}$
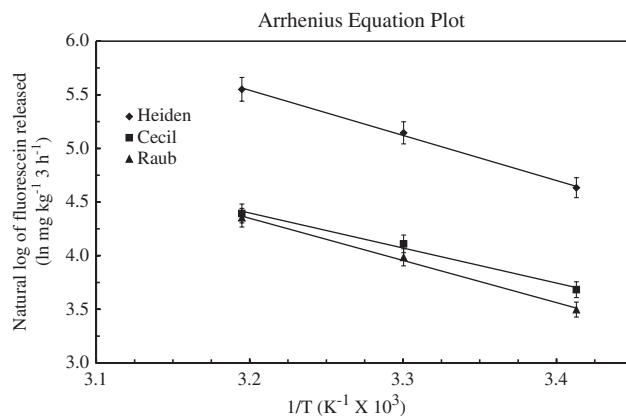

Fig. 7. Arrhenius equation plot of FDA hydrolysis activity values for the Heiden, Cecil, and Raub soils. Points represent the mean of three replicates; error bars represent standard deviation.

constant (K_m) can be determined. By using the Lineweaver–Burke transformation of the Michaelis–Menten equation, we determined K_m and V_{max} values for these soils (Fig. 8). The K_m ranged from 8.3 to 19.9 μM while V_{max} ranged from 64.5 to 250.0 mg fluorescein released kg^{-1} soil 3 h^{-1} (Table 4).

4. Discussion

4.1. Optimization of method

4.1.1. Effect of pH

Previous work to develop a method to assay FDA hydrolytic activity in soil and litter (Schnürer and Rosswall, 1982) began by developing an assay of activity produced in a pure buffer solution adjusted to a pH of 7.6, which was determined to be optimum for most soils. They showed that FDA hydrolysis by pure cultures of *Fusarium culmorum* increased linearly with mycelium
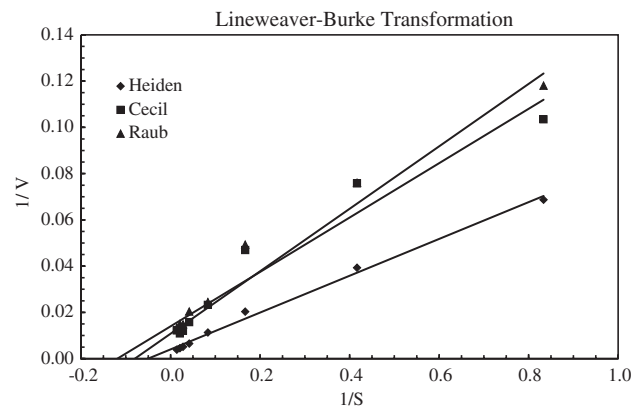

Fig. 8. Presentation of the Lineweaver–Burke transformation of the Michaelis–Menten equation for fluorescein diacetate hydrolytic activity in Heiden, Cecil, and Raub soils.

Table 4
K_m and V_{max} values of FDA hydrolytic activity in soils according to the Lineweaver–Burke transformation of the Michaelis–Menten equation

Soil series	K_m (μM)	V_{max} (mg fluorescein released kg^{-1} soil 3 h^{-1})
Cecil	8.4	71.4
Heiden	19.9	250.0
Raub	8.3	64.5

addition in shaken cultures and after inoculation into sterile soil.

The pH of the Heiden clay soil is 7.8 and this may be why Heiden clay showed optimum activity in a range of alkaline pH, while other soils exhibited optimum activity at pH 7.6. This optimal pH value is also within the 5.5–8.5 pH range that Lundgren (1981) used in studying FDA as a stain for metabolically active bacteria in soil. Fluorescein diacetate has been reported to spontaneously degrade to fluorescein in slightly alkaline (pH$\geq$8.0) solutions (Brunius, 1980). At low pH values ($\leq$5.0), non-biological hydrolysis of FDA may occur (Schnürer and Rosswall, 1982). The effect of pH buffer on FDA hydrolysis is critical because the H$^+$ concentration in the reaction solution affects the ionization groups of the enzyme protein and influences the substrate's ionization state. For effective interaction between the substrate and enzyme, the ionizable groups of both the substrate and the active site of the enzyme must be in their proper states to maintain the correct conformations.

4.1.2. Effect of incubation time

Schnürer and Rosswall (1982) reported a linear relationship between FDA hydrolysis in soils and incubation time (0 to 3 h) based on one soil. Adam and Duncan (2001) show a linear relationship up to 40 min. An incubation time of 3 h allows sufficient time for hydrolysis to take place and provides better differentiation between soils, yet is still not limited by the amount of substrate. Beyond 3 h of incubation, we observed a decrease in the slope of the line, indicating that the substrate may be limiting the reaction beyond 3 h. The observed straight-line relationship up to 3 h indicates that the method developed measures enzymatic hydrolysis of FDA and it is not complicated by microbial growth or assimilation of enzymatic products by microorganisms (Frankenberger and Tabatabai, 1980). Enzyme-catalyzed reactions typically show linear relationships between the amount of products formed and the time of incubation (Deng and Tabatabai, 1994). Skujins (1967) suggests that an assay for soil enzymes should not require incubation times longer than 24 h, due to the risk of error through microbial activity increases with increasing incubation time. The 3 h incubation time chosen fits these guidelines.

4.1.3. Effect of incubation temperature

Schnürer and Rosswall (1982) and Lundgren (1981) used 24 or 22 °C as incubation temperatures, respectively, in their studies of FDA hydrolysis. At 24 °C, however, the distinction between activities of low FDA active soils is quite small. Increasing the temperature to 37 °C allows better differentiation, yet is not too high to cause denaturation. Adam and Duncan (2001), in their assay of FDA, found 30 °C to be the optimum temperature for FDA activity with decrease in activity with temperatures higher than 30 °C. This is different from the data that we present where FDA activity continued to increase up to 60 °C. For environmental samples such as soils, it seems that the enzymes acting in those soils would not be inactivated at soil temperatures commonly encountered during the summer months. However, the differences in optimum temperature may be due to differences in the procedures used.

The FDA hydrolytic enzymes were denatured or inactivated at temperatures above 60 °C. This temperature is similar to that required to denature amidase (Frankenberger and Tabatabai, 1980), arylsulfatase (Tabatabai and Bremner, 1970), inorganic pyrophosphatase (Dick and Tabatabai, 1978), and arylamidase (Acosta-Martinez and Tabatabai, 2000) in soils.

4.1.4. Effect of reaction termination method

Different methods have been used to terminate the FDA hydrolysis reaction. Schnürer and Rosswall (1982) used acetone (50% v/v final concentration) while Adam and Duncan (2001) added 15 ml chloroform/methanol (2:1 v/v) to the 15 ml soil solution. It is important to stop the reaction long enough to measure the absorbance. Our method, using 2 ml acetone, did not completely terminate the reaction, but slowed it sufficiently such that there were no noticeable or significant differences in readings within 30 min of termination. After 30 min, readings began to increase, and differences were significant. This termination of the reaction is similar in effectiveness to that of the procedure of Adam and Duncan (2001) except that their readings decreased with time.

4.1.5. Effects of shaking during incubation

The procedures for several enzyme analyses, including arylamidase (Acosta-Martinez and Tabatabai, 2000) and FDA hydrolysis (Schnürer and Rosswall, 1982; Adam and Duncan, 2001), specify that the samples be shaken during the incubation. Others, such as β-glucosidase (Eivazi and Tabatabai, 1988), aspartase (Senwo and Tabatabai, 1996), arylsulfatase (Tabatabai and Bremner, 1970), acid phosphatase (Eivazi and Tabatabai, 1977) and others specify a static incubation. A static incubation has the advantages of being able to run many samples in a typical incubator and eliminating the need for more expensive environmental shakers. Most importantly, static incubations had higher activity and this becomes important when analyzing soils of low hydrolytic activity.

4.1.6. Effects of substrate concentration

Schnürer and Rosswall (1982) and Lundgren (1981) used a final substrate concentration (concentration of substrate in incubation solution) of 10 µg ml^{-1} (2.9 µM) for measuring FDA hydrolysis in litter and pure cultures, respectively while Adam and Duncan (2001) used a substrate concentration of 67 µg ml^{-1} (32 µM). However, we found that at low substrate concentrations (such as 2.9 or 32 µM), the soils that exhibited higher FDA hydrolytic activity were limited by the amount of substrate available. This was especially true with the Heiden clay soil. Many soil enzyme activities increase with clay content (Dick, 1994) and organic matter (Tabatabai and Bremner, 1970; Trasar-Cepeda et al., 1998); Heiden has a high clay content and high organic matter content. This soil is likely a better representation of a high microbially-active soil than those used by Adam and Duncan (2001) and would likely be substrate limited using that procedure.

4.2. Precision of method

This optimized method was developed for the quantitative determination of FDA hydrolytic activity. If this method is to be used routinely, it is important that the results of the method can be reproduced at different times. The coefficients of variation for the optimized method developed for FDA hydrolytic activity (Table 4) were comparable to other enzymatic activities that have published precision data, such as amidase (Frankenberger and Tabatabai, 1980), L-asparginase (Frankenberger and Tabatabai, 1991a), aspartase (Senwo and Tabatabai, 1996), L-glutaminase (Frankenberger and Tabatabai, 1991b), and invertase (Frankenberger and Johanson, 1983). The range of coefficients of variation for the precision of these enzymes range from 1.0 to 6.6. Precision data has not been published for other methods measuring the hydrolysis of FDA in soil.

4.3. Kinetic parameters

Enzyme catalyzed reactions are temperature dependent (Tabatabai, 1994). While uncatalyzed chemical reactions will approximately double with every increase in 10 °C, enzyme-catalyzed reactions are less sensitive to temperature changes. Generally, an enzyme-catalyzed reaction will increase by a factor <2 for every increase in 10 °C. FDA hydrolysis exhibited a Q_{10} value under 2, which is in line with other enzyme reactions (Table 3).

The activation energy of the catalytic reaction for FDA hydrolysis (Table 3) derived from the Arrhenius plots (Fig. 7) were in line with other published ranges for soil enzymes. L-glutaminase (Frankenberger and Tabatabai, 1991a), L-asparaginase (Frankenberger and Tabatabai, 1991b), and aspartase (Senwo and Tabatabai, 1996) activities in soil exhibit activation energies ranging from 20.3 to 39.9, 20.2 to 34.1 kJ mol^{-1}, and 40.1 to 50.7 kJ mol^{-1}, respectively.

The substrate concentration vs. activity curves for FDA hydrolytic activity in soils obeyed the Michaelis–Menten equation, thus gave a straight line when plotted according to the Lineweaver–Burk transformation (Fig. 8). The Michaelis constant, K_m, calculated from the Lineweaver–Burk transformation represents the substrate concentration at which half the enzyme active sites are filled by substrate molecules and can be equated with the dissociation constant of the enzyme–substrate complex (Chang, 2000). The K_m values we obtained for FDA hydrolysis in soils were lower than other published values: 8.2–33.3 mM for L-glutaminase (Frankenberger and Tabatabai, 1991a), 2.6–8.6 mM for L-asparaginase (Frankenberger and Tabatabai, 1991b), and 173–208 mM for aspartase (Senwo and Tabatabai, 1996). A lower K_m is indicative that FDA hydrolytic enzymes have a high affinity for the substrate.

5. Conclusion

The method we propose for measuring FDA hydrolysis is different in many regards from those previously published. This method has the advantage of using a static incubation, using less solvent to terminate the hydrolysis, and covering a large range of activity. We also include reaction kinetic factors, which have not been determined previously. This method for measuring FDA hydrolysis is simple, sensitive, and precise and should prove useful, especially for studies of soil microbial activity, soil quality, and bioecosystem studies.

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